Impaired Mammary Gland Development and Function in Mice Lacking LAR Receptor-like Tyrosine Phosphatase Activity

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The LAR receptor-like protein tyrosine phosphatase is composed of two intracellular tyrosine phosphatase domains and a cell adhesion molecule-like extracellular region containing three immunoglubulin-like domains in combination with eight fibronectin type-III-like repeats. This architecture suggests that LAR may function in cellular signalling by the regulation of tyrosine phosphorylation through cell–cell or cell–matrix interactions. We used gene targeting in mouse embryonic stem cells to generate mice lacking sequences encoding both LAR phosphatase domains. Northern blot analysis of various tissues revealed the presence of a truncated LAR mRNA lacking the cytoplasmic tyrosine phosphatase domains and indicated that this LAR mutation is not accompanied by obvious changes in the expression levels of one of the LAR-like receptor tyrosine phosphatases PTPδ or PTPσ. LAR$^{-/-}$ mice develop and grow normally and display no appreciable histological tissue abnormalities. However, upon breeding we observed an abnormal neonatal death rate for pups from LAR$^{-/-}$ females. Mammary glands of LAR$^{-/-}$ females were incapable of delivering milk due to an impaired terminal differentiation of alveoli at late pregnancy. As a result, the glands failed to switch to a lactational state and showed a rapid involution postpartum. In wild-type mice, LAR expression is regulated during pregnancy reaching maximum levels around Day 16 of gestation. Taken together, these findings suggest an important role for LAR-mediated signalling in mammary gland development and function. © 1997 Academic Press

INTRODUCTION

   Protein tyrosine phosphorylation, a key event in signal transduction, is a posttranslational modification controlled by the agonistic or antagonistic action of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases, Mourey and Dixon, 1994; Sun and Tonks, 1994). The PTPases comprise a diverse family of receptor-like and cytoplasmic-type enzymes including multiple isoforms re-

sulting from alternative splicing and posttranslational modifications (for review see Saito, 1993).

   Receptor-like PTPases (RPTPases) contain one or two homologous PTPase domains, a transmembrane segment, and diverse combinations of domains with possible ligand-binding properties in the extracellular part. For example, the closely related RPTPases RPTPμ and PTPκ mediate cell–cell interactions in a homophilic, but not heterophilic manner (Brady-Kalnay *et al.,* 1993; Gebbink *et al.,* 1993; Sap *et al.,* 1994; Zondag *et al.,* 1995). In RPTPβ the extracellular moiety binds to a nonrelated cell-surface protein, the neuronal recognition molecule contactin (Peles *et al.,* 1995). It has not been clarified if and how such interactions modulate tyrosine phosphatase activity and whether RPTPases play

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a role early in signal transduction or later in adaptation or response cessation (Ninfa and Dixon, 1994).

Although LAR was among the first RPTPases to be identified (Streuli *et al.,* 1988) and has been studied extensively at the cellular level, its physiological function remains elusive. LAR is composed of two cytoplasmic phosphatase domains in tandem, a transmembrane segment, and an extracellular part which shares homology to immunoglobulin (Ig)-like and fibronectin type III (Fn-III) domains, commonly found in cell adhesion molecules (Edelman and Crossin, 1991). This architecture suggests a physiological function for LAR in cellular signalling by tyrosine dephosphorylation as a response to cell–cell or cell–matrix interactions. Recently, an intracellular LAR-interacting protein (LIP.1) was identified, which binds to the second PTPase domain and colocalizes with LAR to the ends of focal adhesions most proximal to the cell nucleus, suggesting a role for LAR in disassembly of focal adhesions (Serra-Pagès *et al.,* 1995).

LAR has a broad tissue distribution and is expressed on the cell surface as a complex of two noncovalently associated subunits of 150 and 85 kDa, resulting from cleavage of the pro-protein (Streuli *et al.,* 1992; Yu *et al.,* 1992). The N-linked glycosylated 150-kDa fragment is shed during growth. Whether this shedding, which is PMA-inducible (Serra-Pagès *et al.,* 1994), has an effect on the intracellular phosphatase activity remains to be established. The 85-kDa fragment contains a short ectodomain, the transmembrane segment, and the two PTPase domains of ∼280 amino acid residues each. Two closely related family members do exist that show a similar intracellular processing and cell surface expression, namely the LAR-like RPTPases PTPδ and PTPσ (Pulido *et al.,* 1995a; Yan *et al.,* 1993).

In vitro site-directed mutagenesis studies suggested that the first, membrane-proximal phosphatase domain of LAR exhibits catalytic activity, whereas the second, membrane-distal phosphatase domain may have only a regulatory function in modulating the substrate specificity of the first domain (Pot *et al.,* 1991; Streuli *et al.,* 1990). However, the extracellular ligand(s) and downstream substrate(s) of LAR have not been identified. Therefore, we set out to study the consequences of the loss of LAR PTPase function *in vivo* by means of gene targeting using homologous recombination in mouse embryonic stem (ES) cells. Characterization of the resulting LAR$^{-/-}$ mice points to a role for LAR PTPase activity in mammary gland development during pregnancy.

MATERIALS AND METHODS

Generation of LAR-Deficient Mice

The isolation and characterization of mouse 129 genomic LAR sequences were described previously (Schaapveld *et al.,* 1995). A 7.5-kb *Bam*HI–*Eco*RI fragment just upstream of the exons encoding the cytoplasmic phosphatase domains was subcloned into pBluescript KS+ (Stratagene). From this, the 3′ segment was subcloned as a 3.2-kb *Pst*I–*Sal*I fragment into a modified pGEM3 vector having a *Xho*I-linker inserted in the *Sma*I-site, resulting in pLAR5′. From

the 4.5-kb *Bam*HI clone just 448 bp downstream the stop-codon of LAR, the 5′ 2.3-kb *Bam*HI–*Bgl*II fragment was subcloned into the *Bam*HI-site of pLAR5′, generating pLAR5′-3′. Finally, a neomycin phosphotransferase expression cassette (Neo) (van Deursen *et al.,* 1991) was inserted into the *Sal*I-site of pLAR5′-3′. Prior to electroporation, the targeting construct (pLARNeo) was linearized using the unique *Xho*I-site from the introduced linker.

E14 ES cells were cultured on irradiated SNLH9 feeder cells as described (van Deursen and Wieringa, 1992). Exponentially growing ES cells were collected and mixed with 20 μg of linearized pLAR-Neo, electroporated at 250 V/500 μF (Gene Pulser, Bio-Rad), and plated at 5×10^6 per 9-cm dish onto irradiated feeder cells. Selection was applied 24 hr later using 300 μg/ml G418 (Gibco/BRL). After 9–11 days of selection, G418-resistant ES clones were picked and expanded.

Genomic DNA from individual ES cell clones was extracted and analyzed by Southern blot analysis as described (Steeghs *et al.,* 1995). Initial screening was performed using a 3′-flanking probe (a *Sau*3A fragment derived from a 2.5-kb *Bam*HI fragment just downstream of the 4.5-kb *Bam*HI clone mentioned above). Positive clones were also analyzed with a 5′-flanking probe (a 1.0-kb *Bam*HI–*Xba*I fragment representing the 5′ end of the 7.5-kb *Bam*HI–*Eco*RI fragment mentioned above), a 0.8-kb *Pst*I fragment from the Neo cassette, and a 1.8-kb *Bam*HI mouse LAR cDNA fragment comprising all exons encoding both cytoplasmic phosphatase domains (Schaapveld *et al.,* 1995) to check for proper homologous recombination.

Three correctly targeted ES cell clones that also displayed the correct number of 40 chromosomes (CA1, CA7, and BC2) were used for injection into C57BL/6 recipient blastocysts and implanted into the uterine horns of pseudopregnant (C57BL/6 × CBA/Ca)F1 foster mothers (Bradley, 1987). Resulting male chimeras were mated with C57BL/6 females to check for germline transmission. DNA obtained from tail biopsies of agouti offspring and subsequent generations was screened for the presence of the mutant LAR allele by Southern blot analysis and/or polymerase chain reaction (PCR, Hoffmann–La Roche).

Genotyping

A PCR reaction using four primers was designed to discriminate between wild-type (LAR$^{+/+}$), heterozygous (LAR$^{+/-}$), and homozygous mutant (LAR$^{-/-}$) mice. The wild-type allele was detected as a 542-bp amplicon using the primers LAR5s (5′-GAG CTG CGT CAG TTC CA-3′, sense), located 129 bp upstream of the codon for the conserved cysteine residue in the first phosphatase domain, and LAR4as (5′-GAC AGG CTT CCC ACG AC-3′, antisense), a genomic primer located 410 bp downstream of the same cysteine codon. The mutant allele was detected as a 394-bp amplicon using the primers Neo3′ (5′-CTA TCG CCT TCT TGA CGA GTT-3′, sense), located 25 bp upstream of the stop-codon in the Neo cassette, and LAR2as (5′-GAC TTG CTC AGG TAG GGC-3′, antisense), located 532-bp downstream of the stop-codon of LAR. The PCR cycle profile consisted of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min, for 35 cycles, and was performed in a programmable thermocycler (Perkin–Elmer–Cetus). Each 50-μl reaction contained 10 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 2.5 m*M* MgCl$_2$, 0.01% BSA, 0.01% gelatin, 0.05% NP-40, 0.05% Tween-20, 1 m*M* dNTPs, 100 ng genomic DNA, 100 ng of each primer, and 1 U *Taq* polymerase. One-fifth of the reaction products was separated on 2% agarose gels and visualized by ethidium bromide staining.

Breeding of Mice

Mice were kept at the Central Animal Facilty of the University of Nijmegen in a standard room with a day/night rhythm of 06:00/18:00 hr at a temperature of 21°C and a humidity of 50–60%. Males were housed together with four females in a macrolon type I cage and fed *ad libitum.* Upon pregnancy, females were separated to give birth to their pups. The first, second, and third litter of each female was counted and pups were observed every day. Experiments were performed on F2 mice with a 129× C57BL/6 hybrid genetic background. Breeding results were statistically analyzed using MANOVA (SPSS) and Student's *t* test.

RNA Analysis

Poly(A)$^+$ RNA from kidney, brain, heart, liver, and lung (pooled from four 3-months-old male LAR$^{+/+}$, LAR$^{+/-}$, and LAR$^{-/-}$ mice, respectively) was isolated as described previously (Hendriks *et al.,* 1995). For Northern blot analysis, 3 μg of each sample was fractionated on a 1% (w/v) agarose/2.2 M formaldehyde gel, transferred to a Hybond-N$^+$ membrane (Amersham), and linked by UV-radiation (UV Stratalinker 1800, Stratagene). Blots were subsequently probed with the 1.8-kb *Bam*HI mouse LAR cDNA fragment described above, a 162-bp *Eco*RI fragment from mouse LAR cDNA#9 upstream of the deleted region (Schaapveld *et al.,* 1995), a 1.1-kb *Xho*I fragment from the Neo cassette, and mouse cDNA probes (Schaapveld *et al.,* 1995) spanning the phosphatase domains of the LAR-like RPTPases PTPδ (pos. 2486–3815, Mizuno *et al.,* 1993) and PTPσ (pos. 4518-3′ UTR, Ogata *et al.,* 1994), respectively. A rat 1.3-kb *Pst*I glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (Fort *et al.,* 1985) was used to enable comparison of RNA loading. Hybridization signals on autoradiograms were analyzed on a GS-670 imaging densitometer (Bio-Rad). Hybridized probes were stripped for 10 min in boiling 0.1× SSC/0.1% SDS. Removal of probes was checked by exposing filters overnight between hybridizations.

For analysis of LAR expression during mammary gland postnatal development, the first inguinal glands of 3–4 wild-type mice were collected (pregnancy staged by detection of vaginal plug) and RNA was prepared according to Chomzynski and Sacchi (1987). Total RNA (20 μg) was separated on a 1.5% formaldehyde-agarose gel, transferred to Nylon membrane (GeneScreen Plus), and hybridized with the LAR, PTPδ, PTPσ, and GAPDH probes described above, and with a 28S rRNA probe. Phosphor-image analysis was performed using a Model GS-363 Imager System (Bio-Rad) and Molecular Analyst software (Bio-Rad) to quantify the relative expression levels.

Analysis of milk protein expression was done as described previously (Robinson *et al.,* 1995). Briefly, the first inguinal glands of nonlactating LAR$^{-/-}$ and wild-type mice at 18.5 days of pregnancy and at parturition were collected and RNA isolated. For hybridizations, oligonucleotide probes specific for WDNM1, WAP, β-casein, and α-lactalbumin were used.

Histological Analysis

Various tissues from 7-, 14-, and 21-weeks-old mice (male and females) were dissected, fixed in buffered formaldehyde, dehydrated, and embedded in paraffin. Sections of 6-μm thickness were stained with haematoxylin-eosin according to standard histological procedures. Brains were analyzed using parasagittal and coronal sections stained with cresyl violet.

For analyses of mammary glands only female mice with a known history (2–3 litters) regarding neonatal death were used. At the appropriate time point during pregnancy mice were anesthetized with Avertin (Aldrich) and glands were taken surgically. Mice were subsequently allowed to recover and to give birth to their pups. At parturiton and the appropriate time point during the lactation period mice were sacrificed by cervical dislocation.

For histological analyses of mammary glands, the right first inguinal mammary glands were dissected, fixed, and embedded in paraffin as described above. Five-micrometer sections were stained with haematoxylin-eosin or used for immunohistochemical detection of WAP protein as described earlier (Robinson *et al.,* 1995). Mammary glands were also frozen in liquid N$_2$ and embedded in Tissue-Tek O.C.T. Compound (Miles). Serial cryostat-sections of 10 μm were stained with oil-red O to enable detection of lipid vesicles, or with haematoxylin-eosin.

For whole mount preparations of mammary glands the left second thoracic and first inguinal mammary fat pads were dissected, mounted on glass slides, fixed, and stained with Delafield's haematoxylin or carmine as described (Edwards *et al.,* 1988). Subsequently, they were made transparent by immersing in methyl salicylate, analyzed using a dissecting microscope, and photographed. As controls for mouse strain-specific differences, mammary glands from 129, C57BL/6, and (129× C57BL/6)F1 females were included in the analysis.

Milk Protein Analysis

Milk collection was done essentially as described (Simons *et al.,* 1987). At Day 2 of lactation (day of birth of pups is taken as Day 0), female mice were separated from their pups and 2 hr later injected intraperitoneally with 1 or 2 I.U. oxytocin (Intervet). After 10 min milk collection was started.

RESULTS

Targeted Deletion of the LAR Phosphatase Domain Gene Sequences

To study LAR function in an organismal context we used gene targeting by homologous recombination in ES cells to produce LAR$^{-/-}$ mice. A replacement-type targeting vector was constructed in which a positive selection marker, a 1.1-kb neomycin phosphotransferase expression cassette (Neo), is flanked by 3.2 and 2.3-kb of endogenous LAR genomic segments (Schaapveld *et al.,* 1995) which provide the necessary homology for targeted integration. Upon correct homologous recombination, a 4.5-kb genomic segment containing all exons encoding the phosphatase domains of LAR, is replaced by the Neo cassette in the same transcriptional orientation (Fig. 1A). This event results in a shorter *Bam*HI fragment (12.0 to 8.6 kb) and *Eco*RV fragment (20.5 to 12.0 kb) spanning the 5′ and 3′ regions of homology, respectively. This targeting construct was introduced into E14 ES cells by electroporation and 365 G418-resistant clones were screened for homologous recombination. Southern blot analysis using 5′- and 3′-diagnostic probes derived from genomic DNA flanking the targeting vector region revealed a targeting frequency of about 1 in 50 clones. Three cell lines
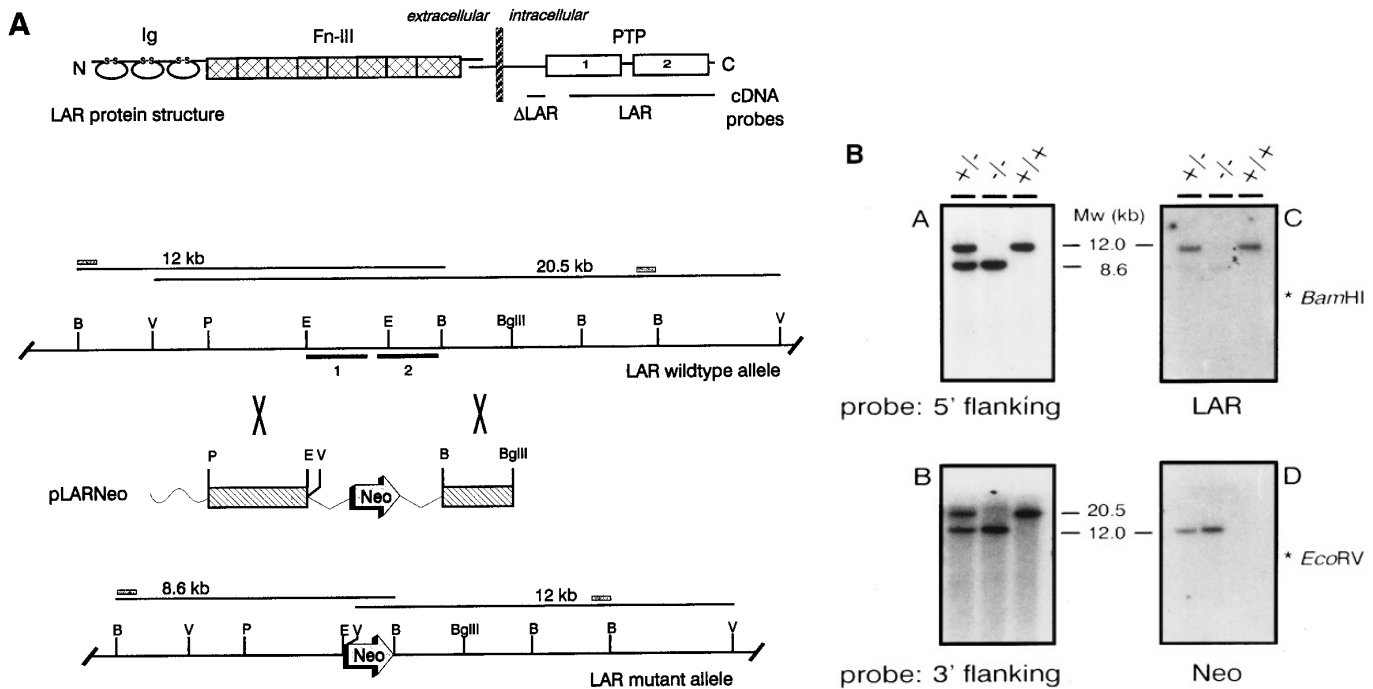
FIG. 1. Disruption of the LAR gene by homologous recombination. (A) LAR protein structure showing the relevant functional domains. Ig, Immunoglobulin-like domains; Fn-III, fibronectin type-III-like domains; PTP, tyrosine phosphatase domains 1 and 2. The LAR cDNA probes as used for Northern blot analysis (see Fig. 2) are indicated by solid bars. Below, a schematic diagram of the relevant part of the mouse genomic LAR locus and the targeting strategy are shown. Hatched bars in pLARNeo represent the fragments homologous to endogenous LAR genomic segments and the gray arrow symbolizes the neomycin phosphotransferase selection cassette. Small gray bars indicate the 5′- and 3′-diagnostic probes, and the solid bars 1 and 2 mark the genomic segments encoding the phosphatase domains 1 and 2, respectively. B, *Bam*HI; E, *Eco*RI: V, *Eco*RV; P, *Pst*I. (B) Southern blot analysis of tail DNA from wild-type (+/+), mice heterozygous (+/−), and mice homozygous (−/−) for the mutant LAR allele. The LAR 5′- and 3′-flanking probes (A and B) reveal the mutant diagnostic 8.6-kb *Bam*HI and 12.0-kb *Eco*RV fragments next to the wild-type 12.0-kb *Bam*HI and 20.5-kb *Eco*RV fragments, respectively. Hybridization with a LAR cDNA probe encompassing both phosphatase domains confirms the total absence of these sequences in LAR$^{−/−}$ mice (C). A Neo cDNA probe reveals the absence of additional integrations of the targeting vector (D).

displaying correct homologous recombination and a normal karyotype were injected into blastocysts. Chimaeric males derived from all three cell lines were mated with C57BL/6 females and shown to transmit the ES cell genome. Offspring carrying the desired mutation were then sibling mated and used to produce heterozygous and homozygous mutant mice in a mixed 129× C57BL/6 background.

To verify the presence of the correct replacement mutation in our lines, offspring born from F1 heterozygote crosses were genotyped by Southern blot (Fig. 1B) or PCR analysis of genomic DNA isolated from tail biopsies. The 5′- and 3′-flanking probes revealed the correct targeting of the LAR locus (Fig. 1B, panels A and B). A LAR cDNA probe encompassing both phosphatase domains (Schaapveld *et al.,* 1995) failed to detect the wild-type 12.0-kb *Bam*HI fragment in DNA isolated from homozygous mutant mice, as anticipated (Fig. 1B, panel C). Using the neomycin-resistance gene as a probe, only the 12.0-kb *Eco*RV fragment and no additional integrations of the targeting construct were detected (Fig. 1B, panel D).

The mutant LAR allele was identified in 145 heterozygous (LAR$^{+/−}$, 49.7%) and 68 homozygous (LAR$^{−/−}$, 23.3%) mice out of a total of 292 mice tested. Thus, the mutant allele segregated essentially according to Mendelian laws, indicating that the removal of the LAR PTPase domains is not lethal.

The Mutant LAR Allele Produces a Truncated mRNA

To investigate the consequences of LAR gene targeting at the RNA level, poly(A)$^+$ RNA was isolated from several tissues and analyzed by Northern blot analysis (Fig. 2). Using the mouse LAR cDNA encompassing both LAR PTPase domains as a probe, no wild-type LAR mRNA was detected in LAR$^{−/−}$ mice (Fig. 2A). The faint signals present in the LAR$^{−/−}$ brain isolate are due to cross-hybridization to the highly homologous RPTPase PTPσ (Schaapveld *et al.,* 1995, see also below). Total absence of LAR mRNA containing the PTPase domains was confirmed by RT-PCR using
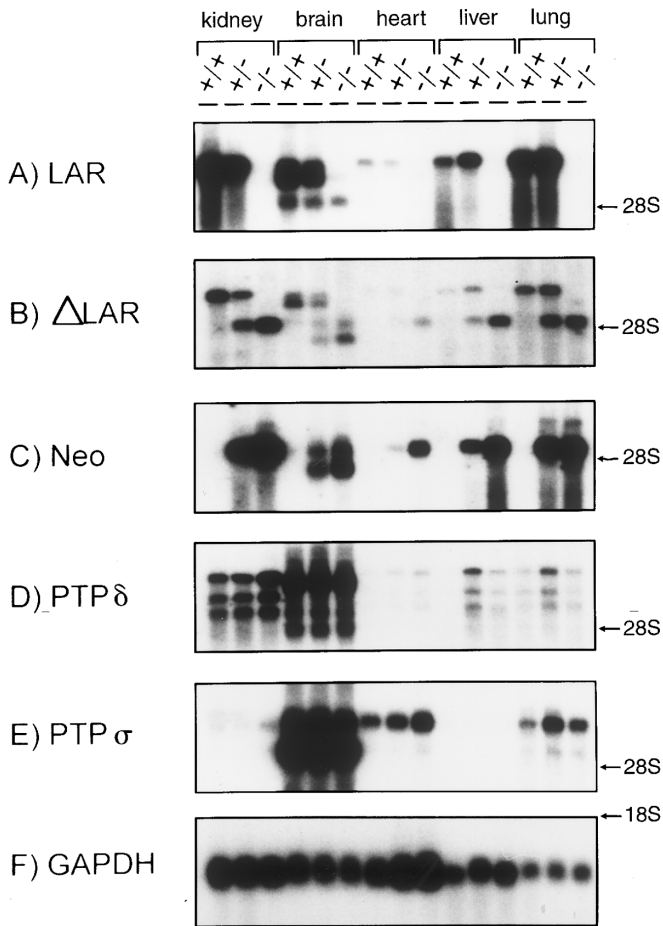
FIG. 2. Northern blot analysis of poly(A)$^+$ RNA isolated from various tissues from LAR$^{+/+}$, LAR$^{+/-}$, and LAR$^{-/-}$ mice using different probes: (A) A mouse LAR cDNA encompassing both PTPase domains of LAR; (B) A mouse LAR cDNA *Eco*RI fragment upstream of the deleted region (ΔLAR); (C) A Neo cDNA *Xho*I fragment; (D) A mouse PTPδ cDNA; (E) A mouse PTPσ cDNA clone; and (F) A rat GAPDH cDNA *Pst*I fragment is used to verify the integrity of the RNA loaded. The positions of the 28S and 18S ribosomal bands are indicated on the right.

mouse LAR-specific primers and poly(A)$^+$ RNA isolated from various tissues (data not shown). In mRNA isolated from wild-type brain, an additional mRNA isoform of ~6.5 kb is seen along with the ubiquitously expressed messenger of ~8 kb.

The mutant LAR allele is expected to give rise to shorter transcripts that contain the sequences encoding the LAR extracellular region and ending at the poly(A) addition site present in the Neo cassette. Indeed both an *Eco*RI mouse LAR cDNA fragment located just upstream of the deleted region and the neomycin-resistance gene probe detect a ~5.5-kb mRNA (Figs. 2B and 2C). In brain a ~4-kb transcript is also found in LAR$^{+/-}$ and LAR$^{-/-}$ mice that likely encodes a shorter LAR isoform lacking extracellular region

sequences (O'Grady *et al.,* 1994; Zhang and Longo, 1995). The truncated transcript(s) in the LAR$^{-/-}$ mice, if translated at all, encode(s) a mutated LAR molecule that lacks PTPase activity.

Histological Analysis of LAR Mutant Mice

Mice lacking the LAR PTPase domains had normal weight, behaved normally and appeared healthy upon veterinary inspection. We then performed an extensive histological survey to look for possible consequences of the absence of functional LAR at the tissue and cellular level. A wide variety of tissues, including brain, kidney, adrenal, liver, lung, heart, stomach, colon, ileum, duodenum, testis, uterus, pancreas, muscle, spleen, thymus, and mammary gland were isolated from 6-, 14-, and 21-week-old male and female mice, sectioned, and stained with haematoxylin-eosin. In addition, serial parasagittal and coronal sections of brain were stained with cresyl violet. This analysis revealed no significant differences between LAR$^{+/+}$, LAR$^{+/-}$, and LAR$^{-/-}$ mice (data not shown).

Expression of LAR-like RPTPases

The LAR-like RPTPases, PTPδ (Mizuno *et al.,* 1993), and PTPσ (Ogata *et al.,* 1994; Wagner *et al.,* 1994), are ~90% identical to LAR at the amino acid level for both PTPase domains (Schaapveld *et al.,* 1995), exhibit similar *in vitro* PTPase activity, and all three interact with the LAR-interacting protein, LIP.1, suggesting similar functions in various tissues (Pulido *et al.,* 1995b). To determine whether PTPδ and/or PTPσ could compensate for LAR function in the LAR$^{-/-}$ mice, we performed Northern blot analysis using mouse cDNA probes encompassing the cytoplasmic regions of PTPδ and PTPσ (Schaapveld *et al.,* 1995). Signal quantitation by densitometry revealed no significant difference in mRNA levels of PTPδ or PTPσ in LAR$^{-/-}$ mice compared with LAR$^{+/-}$ and LAR$^{+/+}$ mice (Figs. 2D and 2E), and the expression distribution of the tissue-specific alternative mRNA isoforms was consistent with data published elsewhere (Mizuno *et al.,* 1993; Ogata *et al.,* 1994; Wagner *et al.,* 1994). This finding cannot rule out any redundancy at the enzymatic level and ultimately crosses between LAR-, PTPδ-, and PTPσ-negative mice will be needed to reveal a potential overlap in function between these RPTPase subfamily members.

Increased Neonatal Mortality for Pups from LAR$^{-/-}$ Females

Unexpectedly upon interbreeding LAR$^{-/-}$ males and females normal-sized litters were obtained, but many of the pups died within 1–3 days after birth. To substantiate this finding, an extensive breeding program was performed in which four possible intercrosses were examined (Fig. 3). We observed an increased neonatal death rate for pups from LAR$^{-/-}$ mothers (>50%) compared to pups from LAR$^{+/+}$
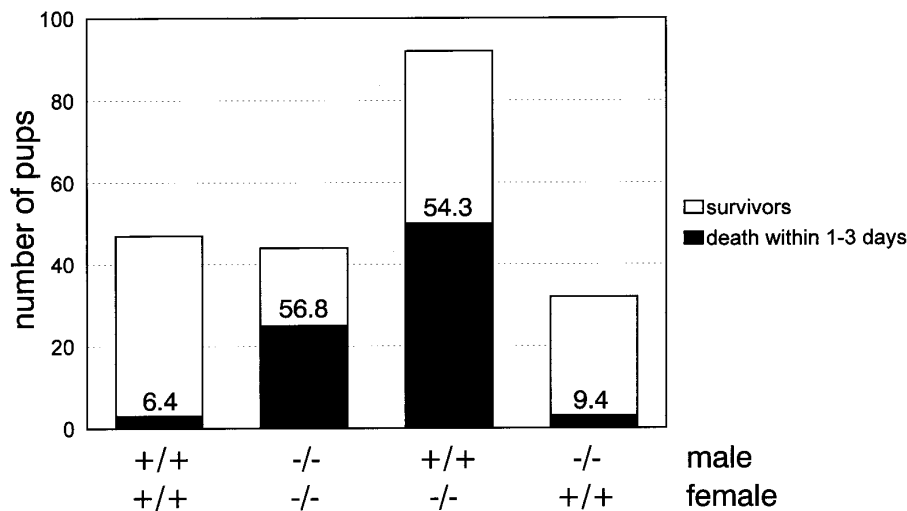
FIG. 3. Increased neonatal mortality for pups of LAR$^{-/-}$ female mice. Upon breeding, LAR$^{-/-}$ females show normal fertility and give birth to normal-sized litters. However, >50% of the pups die within 1–3 days after birth compared to only <10% in the case of LAR$^{+/+}$ females The ratio death/born (%) are indicated reflecting the lack of complete penetrance of the phenotype. Comparable results were found for second and third litters (data not shown). Diagram represents the sum of results with mice derived from the CA1 and BC2 ES cell clones.

mothers (<10%, $t(27,48) = 5.13$, $P < 0.001$), independent of the male genotype and litter size ($F(2,30) = 0.08$, $P = 0.921$). This reduced survival rate was a consistent finding for all three independently derived LAR knock-out lines (originating from ES cell lines CA1, CA7, and BC2) and comparable results were observed for the second and third litters from the same LAR$^{-/-}$ females (data not shown). Ongoing studies using female mice that result from multiple backcrosses onto C57BL/6 background point to full penetrance in C57BL/6 mice with respect to the neonatal death. Thus, most likely the heterogeneity of the genetic background (hybrid 129× C57BL/6) explains the variable penetrance that was observed using F2 animals.

At autopsy, pups born from LAR$^{-/-}$ females which died at Days 1–3 postpartum appeared smaller compared with pups of the same age born from wild-type mice. As indicated by the absence of a white colored belly, no milk was found in their stomachs. Because milk ingestion failed despite multiple attempts to suckle the mothers' nipple, we attribute the neonatal death to a defect in the lactating capacity of the LAR$^{-/-}$ mothers rather than to a behavioral abnormality of neonates. To validate this observation, 1-day-old LAR$^{-/-}$ pups from LAR$^{-/-}$ parents without white bellies were swapped to lactating wild-type mothers. These pups survived demonstrating that their suckling capacity was normal. In contrast, 1-day-old healthy looking LAR$^{+/+}$ pups from wild-type parents were accepted by LAR$^{-/-}$ mothers and did suckle their nipples, but dehydrated and died within 5 days (data not shown).

To investigate a possible milk production defect in LAR$^{-/-}$ mothers in more detail, the quantity of the milk produced by these mice was analyzed. In contrast to lactat-ing LAR$^{+/+}$, LAR$^{+/-}$, or LAR$^{-/-}$ mothers from which normal amounts of milk (20–50 μl) could be readily obtained already at postpartum, we never obtained more than 2–3 μl milk from LAR$^{-/-}$ mothers whose pups died within 1–3 days. Neither the injection of higher doses of oxytocin (2–5 I.U.) nor the intensive massaging of the mammary glands helped to increase milk production (data not shown), suggesting that the lactational defect in LAR$^{-/-}$ mice may be due to a general lack of milk protein synthesis or impaired milk secretion.

Regulation of LAR Expression during Pregnancy

To determine whether LAR has a role in mammary gland development and function, we examined LAR mRNA expression in mammary glands isolated from female mice at various stages during postnatal development. LAR expression is already present in mammary glands of virgin females as determined by RT-PCR (data not shown). Northern blot analysis of total RNA isolated from glands of wild-type females at various stages during pregnancy (P9-18) and at parturition (L1) revealed that LAR expression during pregnancy is developmentally regulated (Fig. 4). LAR expression is evident at day 9 of gestation, increases five-fold during mid-pregnancy and reaches maximum levels at Day 16. Since GAPDH expression might vary during pregnancy, as was found during lactation (Bühler *et al.,* 1993), the variations in the amount of RNA loaded was also assessed by hybridizing the blot with a 28S rRNA cDNA probe. The obtained LAR expression profile is consistent with a role for LAR during mammary gland development at late pregnancy. The expression pattern of the LAR-like RPTPases was also in-
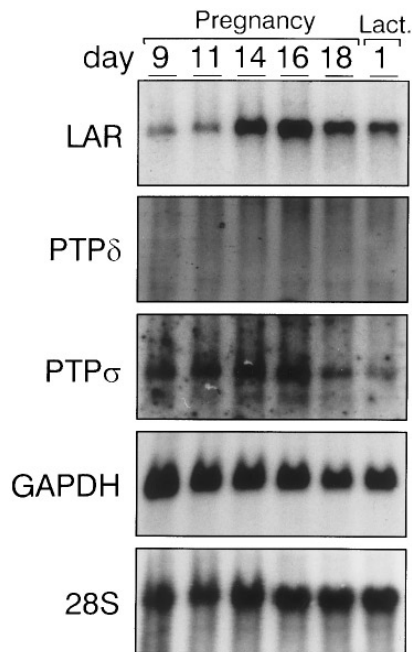
FIG. 4. Regulation of LAR expression during postnatal development of wild-type mammary gland. Total RNA isolated from mouse mammary gland at various stages during pregnancy (P) and at parturition (L, lactating) was analyzed by Northern blotting. The blot was subsequently hybridized with cDNA probes for mouse LAR, PTPδ, PTPσ, rat GAPDH, and 28S RNA (28S). The numbers indicate the stage of gestation (in days) at which the mammary glands were dissected.

vestigated. PTPδ transcripts are undetectable, but PTPσ is expressed, although at very low levels, during mammary gland development (Fig. 4). Compared to LAR, a 20-fold longer exposure time was needed for PTPσ signals to become visible.

Impaired Terminal Differentiation of Mammary Alveoli in Nonlactating LAR$^{-/-}$ Females

To examine whether the lactational defect found in LAR$^{-/-}$ female mice could result from an impaired mammary gland development, we histologically analyzed mammary glands from virgin, late pregnant, postpartum, lactating, and involuting female mice. We termed LAR$^{-/-}$ females "nonlactating" if they consistently showed for two or three successive litters that their pups would die within 1–3 days after birth. LAR$^{-/-}$ females which were indistinguishable from wild-type mice regarding the percentage of surviving pups per litter were considered as "lactating" LAR$^{-/-}$ mice. We observed no significant difference in the number of ducts per unit volume of fat pad in glands of virgin LAR$^{+/+}$, LAR$^{+/-}$, and lactating LAR$^{-/-}$ females (data not shown). Extensive alveolar development was observed dur-

ing lactation in LAR$^{+/+}$, LAR$^{+/-}$, and LAR$^{-/-}$ females which could lactate (Fig. 5B; and data not shown). The lumina were extended and contained secretion. In contrast, less extended alveoli with filled lumina were found in the exceptional case of a nonlactating LAR$^{-/-}$ female which pups survived until Day 7 postpartum (Fig. 5A). The alveolar development in LAR$^{-/-}$ females without the ability to lactate appears incomplete, and the epithelial cells in the alveoli do not have the secretory phenotype and apparently milk production and/or secretion coupled with the formation of an extended lumen was abrogated in nonlactating mice.

The development of mammary glands from nonlactating LAR$^{-/-}$ females up to late pregnancy (Days 18–20) parallels that of wild-type glands, as determined by whole mount analysis (data not shown). Thus, ductal outgrowth and
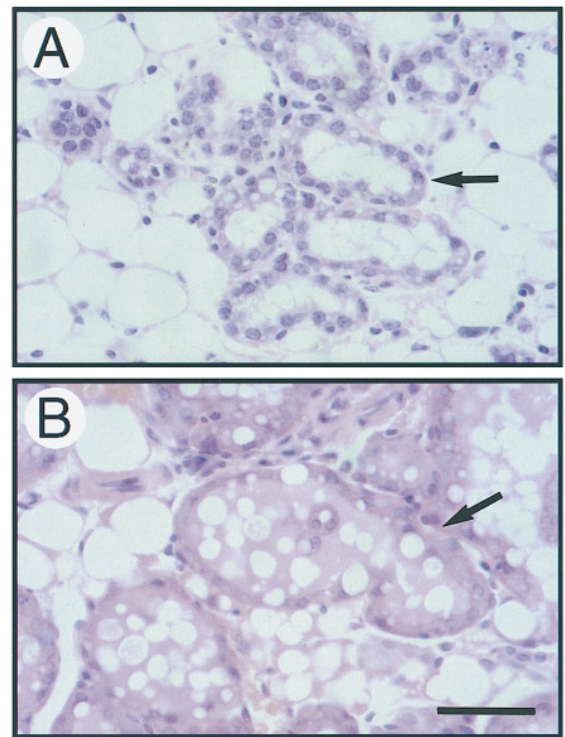

FIG. 5. Histological analysis of mammary tissue from postpartum nonlactating LAR$^{-/-}$ (A) and LAR$^{+/-}$ (B). The former litters of the LAR$^{-/-}$ female died within the first 3 days after birth, but this time the litter was nursed very poorly and the last pups died at Day 7. For the ~50% of females that show the nonlactating phenotype, this was the only exception for which we could monitor a lactational state. The fat pad of the LAR$^{-/-}$ mice is not completely filled and the alveoli are overall smaller than those in hemizygous and wild-type mice (not shown). The epithelial cells in the LAR$^{-/-}$ mice do not have a secretory appearance as witnessed by their cuboidal appearance and flattened apical side (arrow in A). In contrast, milk is visible in the hemizygous mice, both as proteinacious luminal content and as intracellular secretory products (arrow in B). Bar, 200 μm.
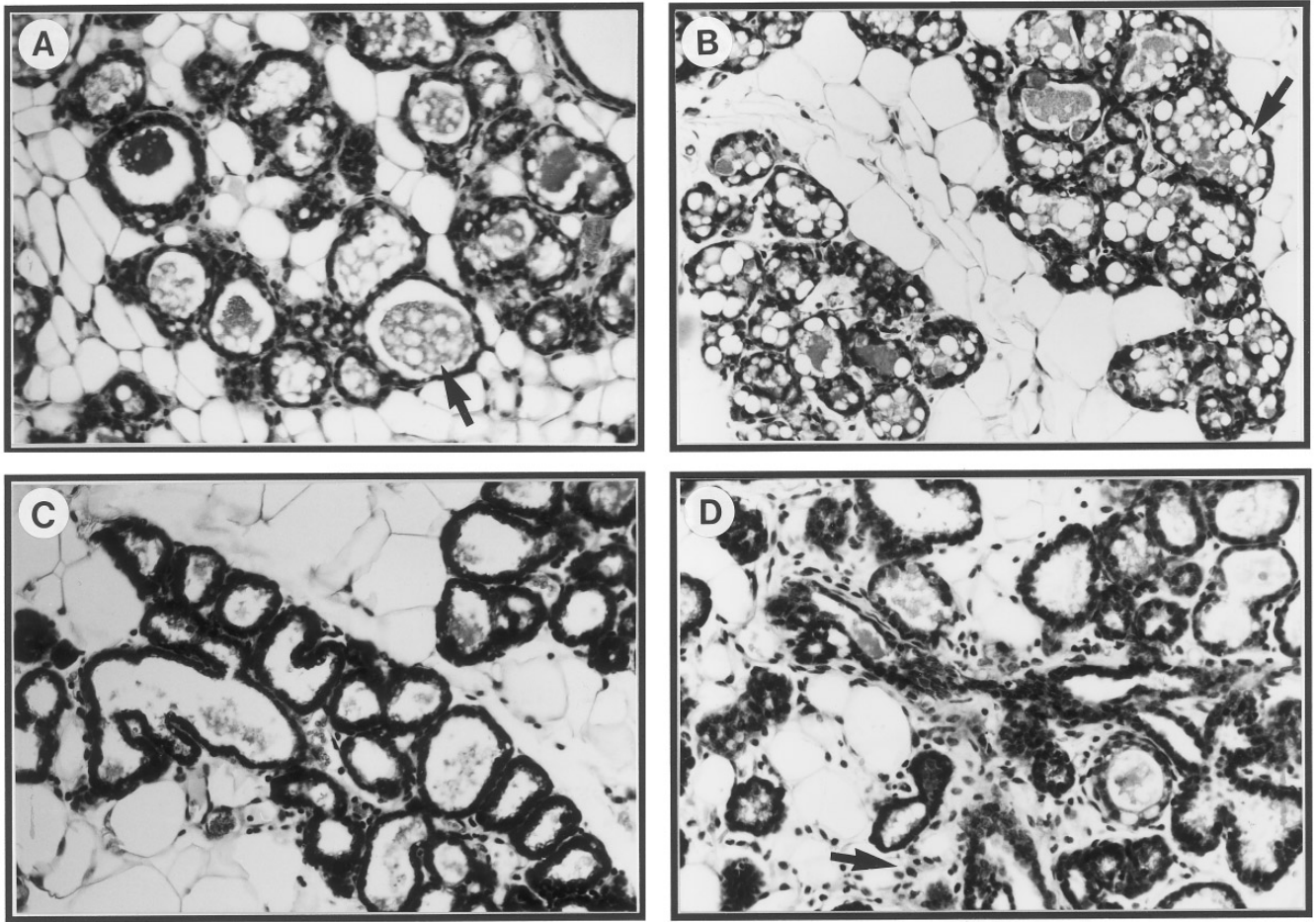
FIG. 6. Aberrant development at late pregnancy resulting in impaired functional differentiation at parturition in LAR$^{-/-}$ mammary glands. Haematoxylin-eosin staining of mammary glands at late pregnancy (Day 18.5; A and B) and at parturition (C and D) from a wild-type female (A and C) and a nonlactating LAR$^{-/-}$ female (B and D). Only minor differences in lumen size and alveoli cluster formation can be observed at late pregnancy. Tissues from wild-type mice contain more intraluminal secretion (arrow in A). In contrast, secretion appears more intracellular in nonlactating LAR$^{-/-}$ glands as indicated by the high content of lipid droplets in epithelial cells (arrow in B). Impaired functional differentiation is clearly visible in glands from nonlactating LAR$^{-/-}$ females at parturition. The number and size of alveoli are reduced. Furthermore, signs of local interstitial fibrosis (arrow in D) and congestion of secretion are visible. Bar, 30 μm.

branching is not overtly impaired. The formation of the lobuloalveolar structures was examined in more detail by histological analysis using haematoxylin-eosin (Figs. 6A and 6B) or oil-red O staining (data not shown) of mammary glands at late pregnancy. Only minor differences in lumen size and alveoli cluster formation were observed between wild-type and nonlactating LAR$^{-/-}$ glands. However, clear differences in the morphology of secretory epithelial cells and luminal contents were evident. While intraluminal secretions were visible in glands from wild-type mice (Fig. 6A), the LAR$^{-/-}$ epithelial cells contained large secretory vesicles and lipid droplets indicating an impaired secretion (Fig. 6B).

At parturition, enlarged alveoli with extended lumina were clearly visible in wild-type glands (Fig. 6C). In contrast, the number and size of alveoli in nonlactating LAR$^{-/-}$ were reduced, and local interstitial fibrosis and congestion of secretion could be observed (Fig. 6D). Whole mount analysis of mammary glands isolated from nonlactating LAR$^{-/-}$ mothers at parturition showed an advanced state of involution (Figs. 7C–F). Although there is unavoidable variation in the moment of tissue collection, the involution state is at least comparable with that of glands isolated from wild-type mice 2 days after removal of their pups (Fig. 7A) and in some cases already resembles complete involution as observed for wild-type mice at 2 weeks following weaning of the pups which had been nursed for three weeks (Fig. 7B).

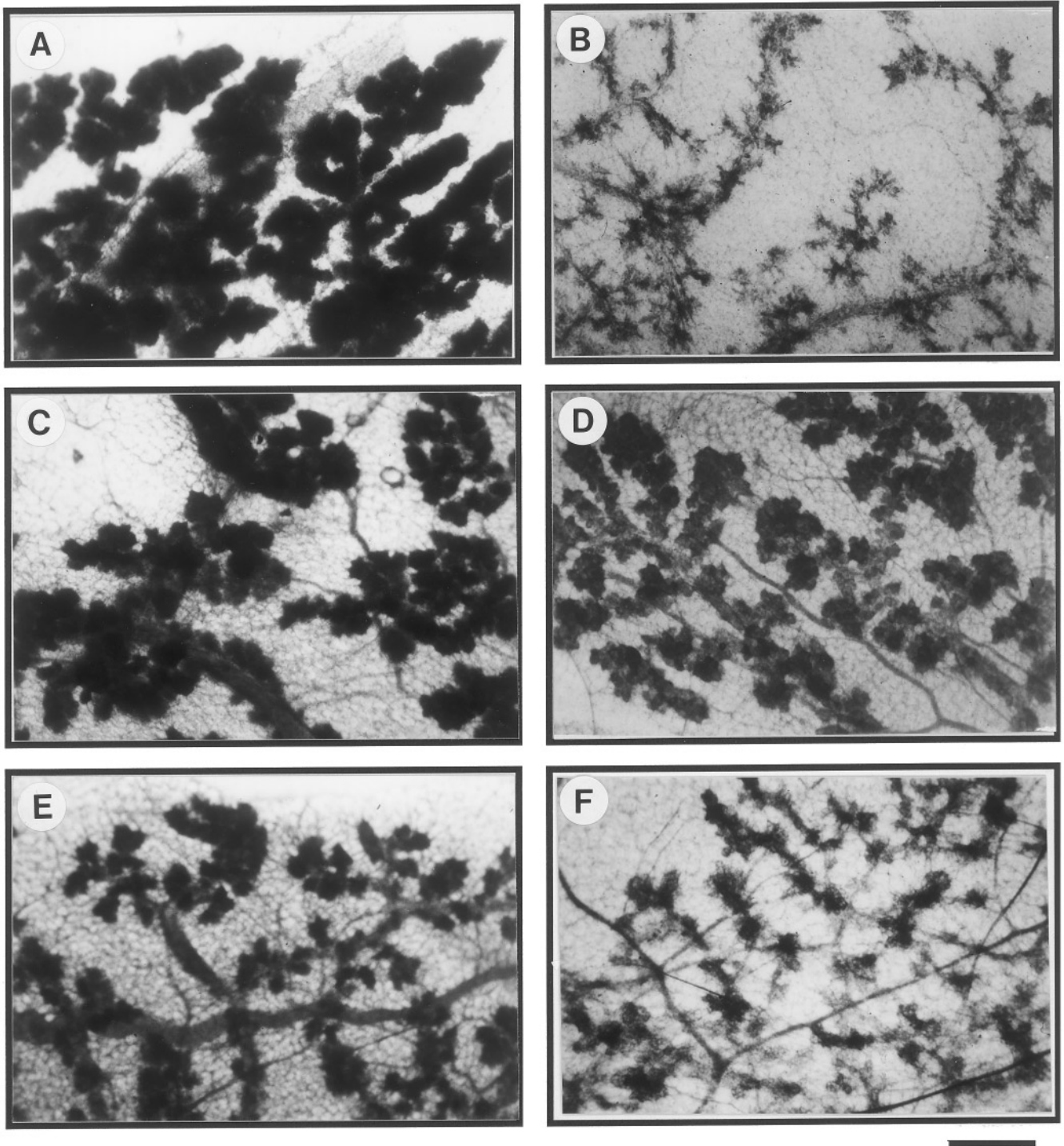To test whether altered expression of milk protein genes

FIG. 7. Rapid involution postpartum in LAR$^{-/-}$ females. Whole mount mammary glands were dissected, fixed, stained with carmine, cleared with methyl salicylate, and photographed. An advanced state of involution is observed in mammary glands from nonlactating LAR$^{-/-}$ mothers at parturition (C–F). For comparison, a mammary gland from a wild-type mother dissected 2 days after removal of pups at parturition (A) and an involuted mammary gland 2 weeks after weaning of pups which were nursed for 3 weeks (B) are shown. Bar, 350 μm.

could explain the failure of nonlactating LAR$^{-/-}$ females to switch to the lactational state, RNA was isolated and probed for WDNM1, WAP, β-casein, and α-lactalbumin transcript levels by Northern blot analysis. No differences were apparent between nonlactating LAR$^{-/-}$ and wild-type mice (data not shown). Also, immmunohistochemical staining for WAP expression did not point to altered milk protein synthesis (data not shown). Moreover, the protein and triglycerides content of the few microliters that incidentally could be obtained from nonlactating mice is comparable to that of wild-type and lactating LAR$^{-/-}$ mice (data not shown).

Pups did recognize, attach, and bite the nipple of their mothers, but in about half of the LAR$^{-/-}$ females alveolar development which is characteristic for late pregnancy was not attained and milk secretion was not initiated. Moreover, the transition to involution and apoptosis of alveolar epithelium occured more readily in these mice, even before milk secretion started. These findings suggest an impaired development of mammary alveoli in LAR$^{-/-}$ females at late pregnancy: the failure of the alveoli to acquire secretory activity is paralleled by a rapid involution at parturition.

DISCUSSION

To address the biological role of LAR PTPase activity in growth and development we generated mice lacking expression of the LAR PTPase cytoplasmic region. The LAR$^{-/-}$ mice are viable, grow, and behave normally. Furthermore, histological analysis of a wide variety of tissues at different ages did not reveal structural abnormalities, consistent with a previous report on the generation of LAR-deficient mice using a gene trap-based method capturing the N-terminal signal sequence and generating a β-galactosidase fusion protein (Skarnes *et al.,* 1995). However, our current detailed analysis reveals that LAR has a function during mammary gland differentiation. We observed an increased neonatal mortality for pups from LAR$^{-/-}$ mothers, independent of the litter size and the genotype of the male or the offspring. The pups suckled the nipples of their mother, but failed to ingest milk, became dehydrated and died. Swapping experiments demonstrated that the increased neonatal death was due to a lactational defect of the LAR$^{-/-}$ mothers rather than to an abnormal behavior of neonatal pups. Due to genetic background heterogeneity this phenotype displayed a 50% penetrance in the F2 hybrid 129× C57BL/6 mice that were used in this study, which might explain why it was not detected by Skarnes *et al.* (1995). Interestingly, also in *Drosophila* the inactivation of CAM-like RPTPases resulted in phenotypes with a limited penetrance (Desai *et al.,* 1996; Krueger *et al.,* 1996). To map potential modifier loci, it will be necessary to cross the LAR allele into different genetic backgrounds. Regarding ductal outgrowth and branching in wild-type mammary glands, we did not observe significant background differences between mouse strain 129, C57BL/6, the F2 hybrid 129× C57Bl/6, and F4 mice on C57BL/6

background, although the ductal system at the virgin state in 129 mice is less developed.

Development of the mammary gland progresses gradually during puberty and pregnancy and acquires full function during lactation (for review see Daniel and Silberstein, 1987). In several natural mouse mutants, as well as transgenic and knock-out mice this process is affected (Gallahan *et al.,* 1996; Jhappan *et al.,* 1992, 1993; Kordon *et al.,* 1995; Li *et al.,* 1994, 1996a,b; Robinson *et al.,* 1996; Sicinski *et al.,* 1995). For example, in transgenic mice expressing TGFα under the control of the mouse WAP gene promoter mammary development and differentiation proceeds during pregnancy, but the secretory cells do not establish and/or maintain a functional state characterized by milk secretion (Robinson *et al.,* 1996; Sandgren *et al.,* 1995). A similar phenotype was observed in mice which contain a nonfunctional mCSF1 (Pollard and Hennighausen, 1994) or inhibin βB gene (Vassalli *et al.,* 1994). The high amounts of intracellular fat droplets at late pregnancy is also seen in ''milchlos'' transgenic mice (Robinson *et al.,* 1995). In nonlactating LAR$^{-/-}$ mice a normal epithelial cell proliferation up to late pregnancy is observed, but histological sections of glands at late pregnancy showed a more intracellular storage of secretion products in comparison with the intraluminal secretion in wild-type glands. At parturition, mammary glands showed a regression of alveolar structures as is seen normally at a progressed stage of involution following weaning of the pups. Taken together, these observations suggest that LAR function is evoked relatively late in mammary gland development and that most likely the LAR$^{-/-}$ phenotype is a combination of a loss of alveolar cells around parturition and the inability of the remaining alveoli to undergo secretory differentiation.

Complex molecular interactions are involved in the regulation of lactation (Rillema, 1994). The initiation of lactation following parturition involves many endocrine changes, of which the reduced influence of sex steroid hormones appears to be the most important (for review see Daniel and Silberstein, 1987; Rillema, 1994). Hormonal influences at the level of some milk protein gene promoters are mediated by the mammary gland-specific transcription factor MGF/Stat5 (Wakao *et al.,* 1994). Regulation of milk protein expression via regulation of Stat5 activity also requires the basement membrane (Sympson *et al.,* 1994; Talhouk *et al.,* 1992), which provides signals via interaction with epithelial cells through cell surface receptors (Streuli *et al.,* 1995). Mice lacking Stat5a show reduced mammary lobuloalveolar outgrowth and fail to lactate due to an impaired terminal differentiation (Liu *et al.,* 1997). The phenotype becomes apparent during midpregnancy and is reflected by a marked reduction in WAP transcript levels. LAR deficient mice show signs of abnormal mammary gland development at a later stage, shortly before parturition, and no reduction in WAP levels could be observed. It remains to be investigated whether or not LAR and STAT5a signalling pathways are intertwined at some stage of mammary gland development.

LAR is expressed in brain and therefore could perhaps play a role in the regulation of the appropriate hormone balance needed for proper mammary gland development and function. However, at the moment it is not known if LAR is specifically localized to regions in the brain that are involved in the hormonal regulation of lactation and further studies will be required to address this issue. LAR is predominantly expressed in epithelial cells and localized at regions of association of these cells and the basement membrane (Streuli *et al.,* 1992). Consistent with this is the recent finding of colocalization of LAR and its interacting protein LIP.1 at focal adhesions and its proposed role in disassembly of focal adhesions (Serra-Pagès *et al.,* 1995). In view of the developmental regulation of LAR expression in the mammary gland during pregnancy and the phenotypic consequences of the ablation of LAR PTPase activity, these data point to an important role for LAR in cell–matrix interactions that are crucial for mammary gland development and function.

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